

Methods in Enzymology

Volume 65

Nucleic Acids

Part I

EDITED BY

Lawrence Grossman

DEPARTMENT OF BIOCHEMISTRY
THE JOHNS HOPKINS UNIVERSITY
SCHOOL OF HYGIENE AND PUBLIC HEALTH
BALTIMORE, MARYLAND

Kivie Moldave

DEPARTMENT OF BIOLOGICAL CHEMISTRY
CALIFORNIA COLLEGE OF MEDICINE
UNIVERSITY OF CALIFORNIA
IRVINE, CALIFORNIA

1980



ACADEMIC PRESS, INC.

(Harcourt Brace Jovanovich, Publishers)

Orlando San Diego New York London
Toronto Montreal Sydney Tokyo

COPYRIGHT © 1980, BY ACADEMIC PRESS, INC.
ALL RIGHTS RESERVED.
NO PART OF THIS PUBLICATION MAY BE REPRODUCED OR
TRANSMITTED IN ANY FORM OR BY ANY MEANS, ELECTRONIC
OR MECHANICAL, INCLUDING PHOTOCOPY, RECORDING, OR ANY
INFORMATION STORAGE AND RETRIEVAL SYSTEM, WITHOUT
PERMISSION IN WRITING FROM THE PUBLISHER.

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data
Main entry under title:

Nucleic acids.

(Methods in enzymology, v. 12, 20-21, 29-30, 65)
Pts. C, E-F have title: Nucleic acids and protein
synthesis: with editor's names in reverse order on t. p.
Includes bibliographical references.
1. Nucleic acids. 2. Protein biosynthesis.
I. Grossman, Lawrence, Date ed. II. Moldave,
Kivie, Date ed. III. Title: Nucleic acids and
protein synthesis. IV. Series: Methods in enzymology.
v. 12 [etc.] [DNLN: 1. Nucleic acids--Biosynthesis.
2. Proteins--Biosynthesis. W 1 Me9615K v. 30 1974
QU 55 N964 1974]
QP601.M49 vol. 12, etc. 574.1'925'08s [QP620]
ISBN 0-12-181965-5 (v. 65) [574.8'732] 74-26909

PRINTED IN THE UNITED STATES OF AMERICA

85 86 87 88

9 8 7 6 5 4 3

CONTRIBUTORS TO
PREFACE
VOLUMES IN SERIES

1. Directory of F

Section :

2. Assaying of
Restriction E
3. Use of Infect
4. Assay for T
Using the E
Duplex Circ
5. Polynucleoti
Class II Res

6. Specific La
Polymerase
7. Terminal
Nucleotides
8. Repair of C
9. Specific La
RNA Ligas

10. 5'-³²P Lab
Fragments

11. General J
Endonucle

12. Purificati

Table of Contents

CONTRIBUTORS TO VOLUME 65	xi
PREFACE	xvii
VOLUMES IN SERIES	xix

1. Directory of Restriction Endonucleases	RICHARD J. ROBERTS	1
---	--------------------	---

Section I. Assays for [Class II Restriction] Endonucleases

2. Assaying of Organisms for the Presence of Restriction Endonucleases	ROBERT SCHLEIF	19
3. Use of Infectious DNA Assays	M. TAKANAMI	23
4. Assay for Type II Restriction Endonucleases Using the <i>Escherichia coli</i> recBC DNase and Duplex Circular DNA	DAVID LACKEY AND STUART LINN	26
5. Polynucleotide Kinase Exchange as an Assay for Class II Restriction Endonucleases	KATHLEEN L. BERKNER AND WILLIAM R. FOLK	28

Section II. Techniques for Labeling Termini

6. Specific Labeling of 3' Termini with T4 DNA Polymerase	MARK D. CHALLBERG AND PAUL T. ENGLUND	39
7. Terminal Transferase-Catalyzed Addition of Nucleotides to the 3' Termini of DNA	RANAJIT ROYCHOUDHURY AND RAY WU	43
8. Repair of Overlapping DNA Termini	HOWARD M. GOODMAN	63
9. Specific Labeling of 3' Termini of RNA with T4 RNA Ligase	T. E. ENGLAND, A. G. BRUCE, AND O. C. UHLENBECK	65
10. 5'- ³² P Labeling of RNA and DNA Restriction Fragments	GEORGE CHACONAS AND JOHAN H. VAN DE SANDE	75

Section III. Purification of Restriction Enzymes

11. General Purification Schemes for Restriction Endonucleases	VINCENZO PIRROTTA AND THOMAS A. BICKLE	89
12. Purification and Properties of <i>Eco</i> RI Endonuclease	ROBERT A. RUBIN AND PAUL MODRICH	96

13. Purification and Properties of <i>Hind</i> II and <i>Hind</i> III Endonucleases from <i>Haemophilus influenzae</i> Rd	HAMILTON O. SMITH AND GARRY M. MARLEY	104
14. Purification and Properties of the <i>Bsp</i> Endonuclease	PÁL VENETIANER	109
15. Purification and Properties of the <i>Bsu</i> Endonuclease	SIERD BRON AND WOLFRAM HÖRZ	112
16. Purification and Properties of the <i>Bgl</i> II and II Endonucleases	THOMAS A. BICKLE, VINCENZO PIRROTTA, AND ROLAND IMBER	132
17. Purification and Properties of the Complementary Endonucleases <i>Dpn</i> I and <i>Dpn</i> II	SANFORD A. LACKS	138
18. Purification and Properties of the <i>Bam</i> HI Endonuclease	GARY A. WILSON AND FRANK E. YOUNG	147
19. Preparation and Properties of the <i>Hpa</i> I and <i>Hpa</i> II Endonucleases	JEROME L. HINES, THOMAS R. CHAUNCEY, AND KAN L. AGARWAL	153
20. Purification and Properties of the <i>Hph</i> I Endonuclease	DENNIS G. KLEID	163
21. Purification and Properties of the <i>Bst</i> 1503 Endonuclease	JAMES F. CATTERALL AND N. E. WELKER	167
22. Purification and Properties of the <i>Sst</i> I Endonuclease	ALAIN RAMBACH	170
23. Preparation and Properties of Immobilized Sequence-Specific Endonucleases	YAN HWA LEE, ROBERT BLAKESLEY, LEONARD A. SMITH, AND JACK G. CHIRIKJIAN	173

Section IV. Purification and Properties of Enzymes Acting at Sites with Altered Bases

24. Purification and Properties of Pyrimidine Dimer-Specific Endonucleases from <i>Micrococcus luteus</i>	SHEIKH RIAZUDDIN	185
25. Purification and Properties of a Pyrimidine Dimer-Specific Endonuclease from <i>E. coli</i> Infected with Bacteriophage T4	ERROL C. FRIEDBERG, ANN K. GANESAN, AND PATRICIA C. SEAWELL	191
26. Exonuclease III of <i>Escherichia coli</i> K-12, an AP Endonuclease	STEPHEN G. ROGERS AND BERNARD WEISS	201
27. Endonuclease IV from <i>Escherichia coli</i>	SIV LJUNGQUIST	212
28. Purification and Properties of the Human Placental Apurinic/Apyrimidinic Endonuclease	NANCY L. SHAPER AND LAWRENCE GROSSMAN	216

29. Purification and Properties of Endodeoxyribonuclease
--

30. Purification and Properties of an Endonuclease Specific for Nonreplicating DNA Induced by Ultraviolet Light

31. The Use of DNA Microarrays for the Study of DNA-Protein Interactions
--

32. Purification and Properties of an Endonuclease from <i>Aspergillus</i>
--

33. Purification and Properties of an Endonuclease from <i>crassa</i> Endonuclease Be Converted to an Exonuclease

34. Purification and Properties of an Endonuclease
--

35. Purification and Properties of an Endonuclease
--

36. Uracil-DNA Glycosylase

37. Purification and Properties of an Endonuclease DNA Glycosylase
--

Section V.

38. Fractionation of Linear RNA in Polyacrylamide Gels or 7 M Formamide

39. Separation and Isolation of Linear Polyacrylamide Gels by Electrophoresis

40. Use of Preparative Gel Electrophoresis for Fragment Isolation

41. RPC-5 Column Chromatography of DNA Fragments
--

42. Fractionation of DNA by Gel Electrophoresis Induced by Polyacrylamide

TABLE OF CONTENTS

vii

SMITH AND RLEY	104
VER	109
ND RZ	112
ICKLE, ROTTA, IMBER	132
LACKS	138
SON AND UNG	147
NES, LAUNCEY, AGARWAL	153
EID	163
TERALL ELKER	167
CH	170
E, ESLEY, SMITH, AND IKJIAN	173

Acting at Sites

DDIN	185
EDBERG, SAN, AND EAWELL	191
OGERS AND SS	201
ST	212
APER AND ROSSMAN	216

29. Purification and Properties of <i>Escherichia coli</i> Endodeoxyribonuclease V	BRUCE DEMPLE, FREDERICK T. GATES III, AND STUART LINN	224
30. Purification and Properties of an Endonuclease Specific for Nonpyrimidine Dimer Damage Induced by Ultraviolet Radiations	SHEIKH RIAZUDDIN	231
31. The Use of DNA Fragments of Defined Sequence for the Study of DNA Damage and Repair	WILLIAM A. HASELTINE, CHRISTINA P. LINDAN, ALAN D. D'ANDREA, AND LORRAINE JOHNSRUD	235
32. Purification and Properties of S_1 Nuclease from <i>Aspergillus</i>	VOLKER M. VOGT	248
33. Purification and Properties of <i>Neurospora crassa</i> Endo-exonuclease, an Enzyme which Can Be Converted to a Single-Strand Specific Endonuclease	M. J. FRASER	255
34. Purification and Properties of the Mung Bean Nuclease	M. LASKOWSKI, SR.	263
35. Purification and Properties of Venom Phosphodiesterase	M. LASKOWSKI, SR.	276
36. Uracil-DNA Glycosylase from <i>Escherichia coli</i>	TOMAS LINDAHL	284
37. Purification and Properties of 3-Methyladenine-DNA Glycosylase from <i>Escherichia coli</i>	SHEIKH RIAZUDDIN	290

Section V. Endonuclease Cleavage Mapping Techniques

38. Fractionation of Low Molecular Weight DNA or RNA in Polyacrylamide Gels Containing 98% Formamide or 7 M Urea	TOM MANIATIS AND ARGIRIS EFSTRATIADIS	299
39. Separation and Isolation of DNA Fragments Using Linear Polyacrylamide Gradient Gel Electrophoresis	PETER G. N. JEPPESEN	305
40. Use of Preparative Gel Electrophoresis for DNA Fragment Isolation	MARSHALL H. EDGELL AND FRED I. POLSKY	319
41. RPC-5 Column Chromatography for the Isolation of DNA Fragments	R. D. WELLS, S. C. HARDIES, G. T. HORN, B. KLEIN, J. E. LARSON, S. K. NEUENDORF, N. PANAYOTATOS, R. K. PATIENT, AND E. SELSING	327
42. Fractionation of DNA Fragments by Polyethylene Glycol Induced Precipitation	JOHN T. LIS	347

43. A Photographic Method to Quantitate DNA in Gel Electrophoresis	ARIEL PRUNELL	353
44. Two-Dimensional Agarose Gel Electrophoresis "SeaPlaque" Agarose Dimension	RICHARD C. PARKER AND BRIAN SEED	358
45. The Use of Intensifying Screens or Organic Scintillators for Visualizing Radioactive Molecules Resolved by Gel Electrophoresis	RONALD A. LASKEY	363
46. Recovery of DNA from Gels	HAMILTON O. SMITH	371
47. The Analysis of Nucleic Acids in Gels Using Glyoxal and Acridine Orange	GORDON G. CARMICHAEL AND GARY K. MCMASTER	380
48. A General Method for Defining Restriction Enzyme Cleavage and Recognition Sites	NIGEL L. BROWN AND MICHAEL SMITH	391
49. Rapid DNA Isolations for Enzymatic and Hybridization Analysis	RONALD W. DAVIS, MARJORIE THOMAS, JOHN CAMERON, THOMAS ST. P. JOHN, STEWART SCHERER, AND RICHARD A. PADGETT	404

Section VI. Determination of DNA Fragment Sizes

50. Conversion of Circular DNA to Linear Strands for Mapping	RICHARD C. PARKER	415
--	-------------------	-----

Section VII. Determination of Fragment Ordering

51. Denaturation Mapping	SANTANU DASGUPTA AND ROSS B. INMAN	429
52. Genetic Rearrangements and DNA Cleavage Maps	A. I. BUKHARI AND D. KAMP	436
53. Determination of Fragment Order through Partial Digests and Multiple Enzyme Digests	KATHLEEN J. DANNA	449
54. Mapping Viral mRNAs by Sandwich Hybridization	ASHLEY R. DUNN AND JOSEPH SAMBROOK	468
55. 5' Labeling and Poly(dA) Tailing	P. G. BOSELEY, T. MOSS, AND M. L. BIRNSTIEL	478

Section

56. RNA Polymerase
57. Sequencing End-Labeled Chemical Cleavage
58. DNA Sequence Analysis
59. Determination of Directed Synthesis Transcripts
60. Computer Analysis
61. Chemical Synthesis of the Modified Tries
62. Sequence Analysis
63. ^3H and ^{32}P Derivation and Sequence
64. A Micromethod for High Molecular Weight
65. Use of <i>E. coli</i> for the Synthesis of Defined Sequence

Section IX.

66. Mapping the Origin of Simian Virus
67. Electron Microscopy Origin and Termination
68. Transcription Mapping RNA: Analysis by Gel Mapping
69. Transcription Mapping
70. Definition and Determination of Transcription

TABLE OF CONTENTS

ix

Section VIII. Nucleotide Sequencing Techniques

56. RNA Polymerase Nascent Product Analysis	M. TAKANAMI	497
57. Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages	ALLAN M. MAXAM AND WALTER GILBERT	499
58. DNA Sequence Analysis by Primed Synthesis	ANDREW J. H. SMITH	560
59. Determination of RNA Sequences by Primer Directed Synthesis and Sequencing of their cDNA Transcripts	P. K. GHOSH, V. B. REDDY, M. PIATAK, P. LEBOWITZ, AND S. M. WEISSMAN	580
60. Computer Analysis of Nucleic Acids and Proteins	CARY L. QUEEN AND LAURENCE JAY KORN	595
61. Chemical Synthesis of Deoxyoligonucleotides by the Modified Triester Method	S. A. NARANG, R. BROUSSEAU, H. M. HSIUNG, AND J. J. MICHNIEWICZ	610
62. Sequence Analysis of Short DNA Fragments	CHEN-PEI D. TU AND RAY WU	620
63. ³ H and ³² P Derivative Methods for Base Composition and Sequence Analysis of RNA	KURT RANDERTH, RAMESH C. GUPTA, AND ERIKA RANDERTH	638
64. A Micromethod for Detailed Characterization of High Molecular Weight RNA	FINN SKOU PEDERSEN AND WILLIAM A. HASELTINE	680
65. Use of <i>E. coli</i> Polynucleotide Phosphorylase for the Synthesis of Oligodeoxyribonucleotides of Defined Sequence	SHIRLEY GILLAM AND MICHAEL SMITH	687

Section IX. Localization of Functional Sites on Chromosomes

66. Mapping the Origin and Terminus of Replication of Simian Virus 40 DNA by Pulse Labeling	CHING-JUH LAI	705
67. Electron Microscopic Methods for Locating the Origin and Termination Points for DNA Replication	GEORGE C. FAREED AND HARUMI KASAMATSU	709
68. Transcription Maps of Polyoma Virus-Specific RNA: Analysis by Two-Dimensional Nuclease S1 Gel Mapping	JENNIFER FAVALORO, RICHARD TREISMAN, AND ROBERT KAMEN	718
69. Transcription Maps of Adenovirus	PHILLIP A. SHARP, ARNOLD J. BERK, AND SUSAN M. BERGET	750
70. Definition and Mapping of Adenovirus 2 Nuclear Transcription	JOSEPH R. NEVINS	768

71. Restriction Fragments from <i>Chlamydomonas</i> Chloroplast DNA	J. D. ROCHAIX	785
72. Template Function of Restriction Enzyme Fragments of Phage M13 Replicative Form DNA	RUUD N. H. KONINGS	795
73. Mapping Simian Virus 40 Mutants by Marker Rescue	CHING-JUH LAI	811
74. Microinjection of Early SV40 DNA Fragments and T Antigen	A. GRAESSMANN, M. GRAESSMANN, AND C. MUELLER	816
75. Assay of Transforming Activity of Tumor Virus DNA	A. J. VAN DER EB AND F. L. GRAHAM	826
76. Bacteriophage λ Repressor and cro Protein: Interactions with Operator DNA	ALEXANDER D. JOHNSON, CARL O. PABO, AND ROBERT T. SAUER	839
77. The Isolation and Properties of CAP, the Catabolite Gene Activator	GEOFFREY ZUBAY	856
78. Lactose Operator-Repressor Interaction: Use of Synthetic Oligonucleotides in Determining the Minimal Recognition Sequence of the Lactose Operator	CHANDER P. BAHL, RAY WU, AND SARAN A. NARANG	877
79. Electron Microscopy of Proteins Bound to DNA	ROBERT SCHLEIF AND JAY HIRSH	885
AUTHOR INDEX		897
SUBJECT INDEX		920

- KAN L. AGARWAL (19), *Dept Biochemistry, University of Chicago, Illinois 60637*
- CHANDER P. BAHL (78), *Cetus Co, Berkeley, California 94710*
- SUSAN M. BERGET (69), *Department chemistry, Rice University, Texas 77001*
- ARNOLD J. BERK (69), *Department biology, University of California, Angeles, California 90024*
- KATHLEEN L. BERKNER (5), *Dep Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts*
- THOMAS A. BICKLE (11, 16), *Dep Microbiology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland*
- M. L. BIRNSTIEL (55), *Institut für kulturelle Biologie II der Universität Höggerberg, 8093 Zürich, Switzerland*
- ROBERT BLAKESLEY (23), *Betsearch Laboratory, Rockville, Maryland 20850*
- P. G. BOSELEY (55), *Department of Sciences, University of Coventry CV4 7A1, United Kingdom*
- SIERD BRON (15), *Department of Centre of Biological Sciences, University of Groningen, 9751 NN Houtkerk 30, The Netherlands*
- R. BROUSSEAU (61), *Division of Sciences, National Research Council, Ottawa, Ontario K1A 0R6, Canada*
- NIGEL L. BROWN (48), *Department chemistry, University of Bristol Medical School, Bristol BS8 1TH*
- A. G. BRUCE (9), *Department chemistry, University of Illinois, Urbana, Illinois 61801*
- A. I. BUKHARI (52), *Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724*

3 gene function, were
segment of Mu DNA.^{23,24}

sequences is the pres-
ents produced by a
cut in the duplicated
duplicated sequences,
n is produced. Let us
am. Tn9 is a transpo-
s at each end the 800
about 1600 base pairs
s present in duplicated
following reasons.²⁴ Two
d by the enzyme *BalI*,
re present in at least
only one copy of Tn9
e only two such frag-
se pairs in length and

gment is generated by

eneral recombination
to inversion. The fre-
be readily detected in

high frequency by site
ent of DNA is present

invertible segments of
apparently controlling

Plasmids and Episomes"
Harbor Laboratory, Cold

phase variation in *Salmonella*,²⁷ the herpes simplex genome²⁸ and the 2 micron circle in *Saccharomyces cerevisiae*.²⁹ A diagnostic feature of flip-flop is the presence of less than stoichiometric amounts of fragments produced by a restriction site located asymmetrically in the invertible segment and a site located outside of the invertible segment. This principle is illustrated by the fragments generated from bacteriophage Mu DNA by the enzymes which cut within and outside of the invertible G segment.^{9,30,31} Figure 7 shows the *KpnI*-*PstI* digests of Mu DNAs. *KpnI* site is located asymmetrically within G whereas the *PstI* site is outside of G. Two different fragments can be seen when the DNA molecules have the G segment in both orientations. When G is only in one orientation, only one fragment specific to a particular orientation can be seen.

²⁷ J. Zieg, M. Silverman, M. Hilmen, and M. Simon, in "The Operon" (J. H. Miller and W. S. Reznikoff, eds.), p. 411. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1978.

²⁸ B. Roizman, *Cell* 16, 481 (1979).

²⁹ J. R. Broach, J. F. Atkins, C. McGill and L. Chow, *Cell* 16, 827 (1979).

³⁰ D. Kamp, R. Kahmann, D. Zipser, T. R. Broach, and L. T. Chow, *Nature (London)* 271, 577 (1978).

³¹ A. Toussaint, N. Lefebvre, J. R. Scott, J. A. Cowan, F. DeBruijn, and A. I. Bukhari, *Virology* 89, 146 (1978).

³² M. Magazin, M. Howe, and B. Allet, *Virology* 77, 677 (1977).

[53] Determination of Fragment Order through Partial Digests and Multiple Enzyme Digests

By KATHLEEN J. DANNA

Many applications of restriction endonuclease cleavage of DNA are possible only if the resulting fragments have been ordered to produce a physical map. This article describes the basic principles of two techniques for fragment ordering: analysis of partial digestion products and multiple enzyme digestion. These were the first methods used to determine a physical map of a DNA genome, the simian virus 40 (SV40) genome,¹ and remain perhaps the most straightforward and easy to interpret procedures. Moreover, aside from apparatus for DNA fragment analysis, these approaches require only the DNA and restriction endonuclease(s) of interest.

Ordering DNA fragments by partial endonuclease digestion is analogous to a sequencing technique for RNA described by Adams *et al.*,² who

¹ K. J. Danna, G. H. Sack, Jr., and D. Nathans, *J. Mol. Biol.* 78, 363 (1973).

² J. M. Adams, P. G. N. Jeppesen, F. Sanger, and B. G. Barrell, *Nature (London)* 223, 1009 (1969).

used partial T1 ribonuclease digestion products of R17 RNA to order T1 oligonucleotides of the limit digest. A partial digest of DNA is obtained by limiting the reaction time so that the endonuclease does not cleave at all possible recognition sites in the DNA. Thus, partial digestion yields some fragments comprised of two or more contiguous complete digestion products. By purifying a partial digestion product, incubating it with excess enzyme to complete the digestion, and identifying the resultant fragments, one can determine which final products are contained in a given partial digestion product. Analysis of several partial digestion products in this way enables one to deduce the order of all fragments of the limit digest.

Multiple enzyme digestion for ordering DNA fragments employs an approach routinely used for sequencing proteins and RNA, namely, sequential digestion with enzymes of different specificity. For DNA, the cleavage products of one endonuclease are characterized with respect to size and are then digested with a second endonuclease. Analysis of the resultant double-digestion products establishes the relationship between the cleavage sites of the two enzymes.

The partial digest and multiple enzyme digest approaches to fragment ordering are best illustrated by example. This chapter presents a model study which develops a physical map of the SV40 genome. Section I describes procedures for digestion of DNA with endonuclease and for analysis of cleavage products, with emphasis on techniques, such as polyacrylamide gel electrophoresis and autoradiography, that are used in the model study. Ordering fragments through analysis of partial digestion products is illustrated in Section II for the two sets of SV40 DNA fragments produced by cleavage with *Hinc*II and with *Hind*III. In Section III, *Taq*I and *Bam*H1 are used in multiple enzyme digestions with *Hinc*II and *Hind*III to generate a complex physical map that includes the cleavage sites for all four enzymes.

I. Basic Procedures

A. Digestion of DNA with Restriction Endonucleases

The first step in ordering DNA fragments is complete digestion of DNA with the endonuclease(s) of choice. Optimal reaction conditions (e.g., pH, salt concentrations, and temperature) for specific restriction endonucleases are described in the catalogues published by suppliers³ and

³ Bethesda Research Laboratories, Rockville, Maryland; Boehringer Mannheim, Indianapolis, Indiana; Miles Laboratories, Inc., Elkhart, Indiana; New England Biolabs, Beverly, Massachusetts.

elsewhere in the volume. A partial digest of DNA is obtained by limiting the reaction time so that the endonuclease does not cleave at all possible recognition sites in the DNA. Thus, partial digestion yields some fragments comprised of two or more contiguous complete digestion products. By purifying a partial digestion product, incubating it with excess enzyme to complete the digestion, and identifying the resultant fragments, one can determine which final products are contained in a given partial digestion product. Analysis of several partial digestion products in this way enables one to deduce the order of all fragments of the limit digest.

Multiple enzyme digestion for ordering DNA fragments employs an approach routinely used for sequencing proteins and RNA, namely, sequential digestion with enzymes of different specificity. For DNA, the cleavage products of one endonuclease are characterized with respect to size and are then digested with a second endonuclease. Analysis of the resultant double-digestion products establishes the relationship between the cleavage sites of the two enzymes.

The partial digest and multiple enzyme digest approaches to fragment ordering are best illustrated by example. This chapter presents a model study which develops a physical map of the SV40 genome. Section I describes procedures for digestion of DNA with endonuclease and for analysis of cleavage products, with emphasis on techniques, such as polyacrylamide gel electrophoresis and autoradiography, that are used in the model study. Ordering fragments through analysis of partial digestion products is illustrated in Section II for the two sets of SV40 DNA fragments produced by cleavage with *Hinc*II and with *Hind*III. In Section III, *Taq*I and *Bam*H1 are used in multiple enzyme digestions with *Hinc*II and *Hind*III to generate a complex physical map that includes the cleavage sites for all four enzymes.

Preparative digestions that yield fragments of all sizes categorized for different purposes are described in the catalogues published by suppliers³ and elsewhere in the volume. A partial digest of DNA is obtained by limiting the reaction time so that the endonuclease does not cleave at all possible recognition sites in the DNA. Thus, partial digestion yields some fragments comprised of two or more contiguous complete digestion products. By purifying a partial digestion product, incubating it with excess enzyme to complete the digestion, and identifying the resultant fragments, one can determine which final products are contained in a given partial digestion product. Analysis of several partial digestion products in this way enables one to deduce the order of all fragments of the limit digest.

B. Analysis of DNA Fragments

DNA fragments are separated by size using polyacrylamide gel electrophoresis or agarose gel electrophoresis. The fragments are then analyzed by autoradiography or by fluorescence.

⁴ See this volume.

⁵ For example, completely digests.

⁶ R. D. Wells et al.

⁷ D. Davis et al.

⁸ P. A. Sharp, I.

⁹ R. C. Parker et al.

17 RNA to order T1
f DNA is obtained by
does not cleave at all
digestion yields some
complete digestion prod-
ucts by adding excess
resultant fragments,
ed in a given partial
tion products in this
s of the limit digest.
ragments employs an
d RNA, namely, se-
cificity. For DNA, the
rized with respect to
ase. Analysis of the
relationship between

approaches to fragment
ter presents a model
genome. Section I
endonuclease and for
techniques, such as
phy, that are used in
s of partial digestion
of SV40 DNA frag-
ments. In Section III,
ions with *HincII* and
cludes the cleavage

complete digestion of
reaction conditions
specific restriction
ed by suppliers³ and

Bringer Mannheim, In-
New England Biolabs,

elsewhere in this volume.⁴ However, the amount of enzyme⁵ needed to yield a limit digest must be determined empirically because the number of cleavage sites for a particular endonuclease in a given species of DNA cannot be predicted. A series of pilot reactions, in which both the ratio of enzyme to DNA and the incubation time are varied, is useful for determining the amount of enzyme needed to attain complete digestion. In the model study, each pilot reaction contained 0.2 μ g of SV40 DNA in a 20- μ l volume with either 0.1 unit, 0.5 unit, or 1 unit of enzyme. A 5- μ l aliquot was withdrawn from each reaction mixture at 30 min, 1 hr, 2 hr, and 3 hr, and reaction in each was stopped by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1% (w/v). Samples were then analyzed electrophoretically, as described in Section I,B, to determine which conditions resulted in complete digestion.

An important characteristic of a limit digest is that all cleavage products are equimolar. Therefore, when uniformly labeled [³²P]DNA is cleaved, the amount of radioactivity in each limit product is directly proportional to its size. Complete digestion can be verified by the addition of more endonuclease to a reaction mixture and incubation for a longer time. If digestion is complete, neither the amounts nor the sizes of the products will change.

The same methods can be used to establish conditions for partial digestion. Short reaction times result in large fragments that contain several contiguous complete digestion products, and longer times result in smaller fragments. A preparation of partial digestion products including fragments of all sizes can be obtained by combining several reaction mixtures incubated for different lengths of time.

Preparative reaction mixtures should be exactly scaled to pilot reactions that yield a high proportion of the desired products. A preparative digest containing 1×10^6 to 2×10^6 dpm of [³²P]DNA proved sufficient to map SV40 DNA, which is about 5000 nucleotide pairs in length.

B. Analysis of Cleavage Products

DNA fragments produced by restriction endonucleases have been separated by reverse phase chromatography,⁶ hydroxylapatite chromatography,⁷ agarose gel electrophoresis,^{8,9} and polyacrylamide gel

⁴ See this volume, Section III.

⁵ For example, New England Biolabs defines 1 unit of enzyme as the amount that completely digests 1 μ g of phage λ DNA in 15 min at the optimal temperature of incubation.

⁶ R. D. Wells *et al.*, this volume, Article [41].

⁷ D. Davis *et al.*, this volume, Article [49].

⁸ P. A. Sharp, B. Sugden, and J. Sambrook, *Biochemistry* 12, 3055 (1973).

⁹ R. C. Parker and B. Seed, this volume, Article [44].

electrophoresis.^{10,11} For most of the analyses in the model study, vertical slab gels of polyacrylamide were used because of their high resolving power and high capacity. Visualization of DNA fragments in gels has been achieved by the use of both fluorescent¹² and nonfluorescent stains,¹³ by the use of tungstate screens,¹⁴ and by autoradiography.¹⁵ In the model study, fragments of [³²P]DNA (specific activity of 5×10^5 dpm/ μ g of DNA) were visualized by autoradiography, a sensitive method that allows as little as 10^{-3} μ g of DNA to be observed in 16 hr. Detailed descriptions of both slab gel electrophoresis and autoradiography have been presented in this series.^{9-11,14,16} The remainder of this section reviews only the specific techniques used to prepare the slab gels, samples, and wet- and dried-gel autoradiograms for the model study.

Slab gels (14-cm wide, 13-cm long, 1-mm thick) are routinely prepared by the method of Loening¹⁷ from these stock solutions:

1. Acrylamide (recrystallized from ethyl acetate), 15% (w/v)-*N,N'*-methylenebisacrylamide (recrystallized from acetone), 0.75% (w/v)
2. 10 \times electrophoresis buffer: 0.4 *M* Trizma base, 0.2 *M* sodium acetate, 0.02 *M* sodium EDTA, adjusted to pH 7.8 with glacial acetic acid
3. Ammonium persulfate, 5% (w/v), freshly made
4. *N,N,N',N'*-tetramethylethylenediamine (TEMED), neat

For a 4% polyacrylamide gel (total volume 40 ml), 10.7 ml of stock acrylamide solution are mixed with 4 ml of 10 \times electrophoresis buffer and 24.9 ml of deionized water. Polymerization is catalyzed by the addition of 0.42 ml of 5% ammonium persulfate and 0.042 ml of TEMED. The solution is poured between two glass plates, as described by DeWachter and Fiers,¹⁶ to form the slab gel.

Prior to electrophoresis, DNA samples containing 1% SDS (w/v) are incubated at 37° for 10 min to disrupt protein-DNA aggregates. Samples are then made 10% (w/v) in sucrose and 0.02% (w/v) in bromphenol blue, are layered into wells in the gel, and are electrophoresed at constant voltage in a buffer of 0.04 *M* Trizma base, 0.02 *M* sodium acetate, 0.002 *M* sodium EDTA, adjusted to pH 7.8 with glacial acetic acid.

The time of electrophoresis and voltage required depend on the range of fragment sizes that must be resolved. A mixture of DNA fragments

¹⁰ T. Maniatis and A. Efstratiadis, this volume, Article [38].

¹¹ P. G. N. Jeppesen, this volume, Article [39].

¹² G. G. Carmichael and G. K. McMaster, this volume, Article [47].

¹³ G. S. Hayward, *Virology* 49, 342 (1972).

¹⁴ R. A. Laskey, this volume, Article [45].

¹⁵ K. J. Danna and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* 68, 2913 (1971).

¹⁶ R. DeWachter and W. Fiers, Vol. 21, Part D, p. 167.

¹⁷ U. Loening, *Biochem. J.* 102, 251 (1967).

ranging in
13-cm long
hr. For ac
products,
increased
percentag

Autor:
wet-gel ex
by dried-
tion of [³²
toradiogr:
gel, suppo
piece of r
X-omat) i
top of the
time, the
Rapid-Fi:
produces
exposure

The
sharper t
tive samj
or by Ma
The gel i
gel-side
polyethy
gel drye:
plate (ab
covered
when th
uum dri
placed t

C. Purif

Indi
electrop
recover

¹⁸ For ex
Richm

¹⁹ J. V. M

²⁰ G. Fair
393 (19

[53]

ranging in size from 200 to 2000 nucleotide pairs can be resolved on a 13-cm long slab gel of 4% polyacrylamide by electrophoresis at 120 V for 3 hr. For adequate resolution of larger fragments, such as partial digestion products, the voltage or the time of electrophoresis or both should be increased. As an alternative, a gel with a larger pore size (i.e., lower percentage of acrylamide or agarose) can be employed.

Autoradiographic analysis of [32 P]DNA fragments can be achieved by wet-gel exposure of X-ray film, as described by DeWachter and Fiers,¹⁶ or by dried-gel exposure. Wet-gel autoradiography is essential for purification of [32 P]DNA fragments from gels (see Section I,C). For wet-gel autoradiography, one of the glass plates enclosing the gel is removed; the gel, supported by the remaining glass plate, is covered with Saran wrap; a piece of medical X-ray film (e.g., Kodak Blue Brand or Kodak RP Royal X-omat) is laid atop the Saran wrap; and a clean glass plate is clamped on top of the film to ensure uniform contact. After an appropriate exposure time, the film is processed in Kodak D-19 developer (5 min) and Kodak Rapid-Fixer (5 min). As little as 2000 dpm of [32 P]DNA in an area of 1 mm² produces an easily visible spot on Kodak Blue Brand film after a 30-min exposure.

The alternate procedure of dried-gel autoradiography results in sharper bands because of a reduced scattering angle between the radioactive sample and the film. The gel can be dried on an automatic gel dryer¹⁸ or by Maizel's modification¹⁹ of a method described by Fairbanks *et al.*²⁰ The gel is first transferred to a sheet of Whatman 3MM paper, is placed gel-side up on a porous support (either a metal grid or a porous polyethylene sheet), and is covered with Saran wrap. With an automatic gel dryer, the assembly is placed gel-side up onto a prewarmed heating plate (about 80°), which has an integral vacuum manifold. The assembly is covered with a sheet of silicone rubber, which forms a seal about the gel when the vacuum system is activated. The combination of heat and vacuum dries a 14-cm × 13-cm × 1-mm gel in about 35 min. The dried gel is placed tightly against a piece of X-ray film for autoradiography.

C. Purification of DNA Fragments

Individual DNA fragments are conveniently purified by preparative gel electrophoresis, excision of gel segments containing DNA bands,¹⁶ and recovery of the DNA from each segment. For purification of the [32 P]DNA

¹⁸ For example, a Gel Slab Dryer, Model 224, manufactured by Bio-Rad Laboratories, Richmond, California.

¹⁹ J. V. Maizel, Jr., *Methods Virol.* 5, 180.

²⁰ G. Fairbanks, Jr., C. Levinthal, and R. H. Reeder, *Biochem. Biophys. Res. Commun.* 20, 393 (1965).

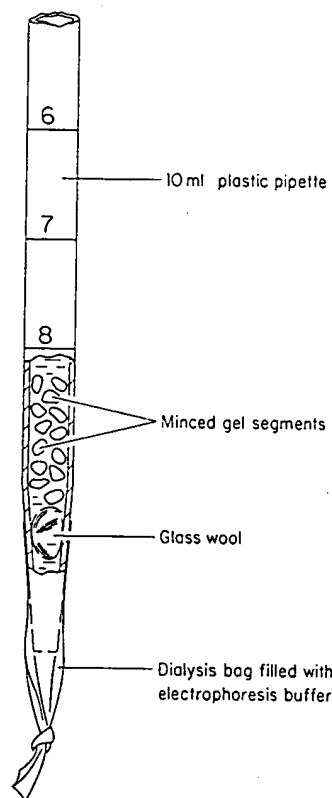


FIG. 1. Apparatus for recovery of DNA from gel segments by electrophoresis into a dialysis bag.

fragments in the model study, a wet-gel exposure of a preparative gel was made as described in Section I,B, except that labels written with ^{32}P -ink were placed at the corners of the slab before exposure.¹⁶ The developed X-ray film was aligned on top of the gel by means of the radioactive labels, and the outline of the gel was traced onto the film. With the guidance of the tracing, the film was accurately aligned under the glass plate supporting the gel so that gel segments corresponding to DNA bands could be excised with a scalpel or razor blade.

For a description of general methods to recover DNA from gels, see Smith.²¹ In the model study, recovery was accomplished by electrophoresis of the sample into a dialysis bag, a reliable method, which results in 80–90% recovery of DNA. A simple apparatus, illustrated in Fig. 1, consists of a short segment of a 10-ml plastic pipette with a glass

²¹ H. O. Smith, this volume, Article [46].

wool plug in
filled with
over the tap
electrophoresis
are transfer
placed into
positive ele
quent elect
directly or
pH 6.0, an
gels in this
model stud
pairs in len
gel segmen

II. Orderir

Analys:
cleavage o
described

1. Th
2. Inc
3. Ea
4. Th

The m
and *Hind*
5226²³ nu
digestion
Because
HincII di
phabetic
from the
(lane c) :

²² W. Fier
Heuvers
113 (197

²³ V. B. Re
Ghosh, I

[53]

wool plug in the tip. Attached to the pipette is a 3–4-cm long dialysis bag filled with electrophoresis buffer. The dialysis tubing should fit tightly over the tapered end of the pipette. After the pipette has been filled with electrophoresis buffer, minced gel segments containing a DNA fragment are transferred into the pipette and allowed to settle. The assembly is placed into a cylindrical gel apparatus with the dialysis bag toward the positive electrode so that the DNA will migrate into the bag during subsequent electrophoresis. The DNA recovered from the bag can be used directly or can be concentrated by precipitation in 0.03 M sodium acetate, pH 6.0, and 70% ethanol at -20°C . DNA purified from polyacrylamide gels in this way is suitable for further endonuclease digestion. In the model study, 90% of a partial digestion product about 1000 nucleotide pairs in length was recovered from a 1-ml volume of 4% polyacrylamide gel segments by electrophoresis at 150 V for 3 hr.

II. Ordering of Fragments by Partial Digestion

Analysis of partial digestion products to order fragments produced by cleavage of DNA with a restriction endonuclease employs the techniques described in Section I in the following steps.

1. The electrophoretic profile for products of complete digestion is established.
2. Individual partial digestion products from a large-scale digest are purified.
3. Each partial digestion product is redigested with an excess of enzyme and electrophoresed in parallel with a marker of completely digested DNA.
4. The resulting data are analyzed to construct a physical map.

The method is exemplified by the mapping of cleavage sites for *HincII* and *HindIII* on SV40 DNA, a circular molecule with a length of 5224²² or 5226²³ nucleotide pairs. Figure 2 shows the major products of complete digestion of SV40 DNA with *HincII* (lane a) and with *HindIII* (lane b). Because they migrated off the gel, the two smallest fragments in the *HincII* digest, F and G, are not shown. The fragments are labeled alphabetically in order of decreasing size, and the length of each, derived from the nucleotide sequence of the DNA, is listed in Table I. Figure 2 (lane c) shows an example of an incomplete digest of SV40 DNA with

²² W. Fiers, R. Contreras, G. Haegeman, R. Rogiers, A. Van de Voorde, H. Van Heuverswyn, J. Van Herreweghe, G. Volckaert, and M. Ysebaert, *Nature (London)* 273, 113 (1978).

²³ V. B. Reddy, B. Thimmappaya, R. Dhar, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman, *Science* 200, 494 (1978).

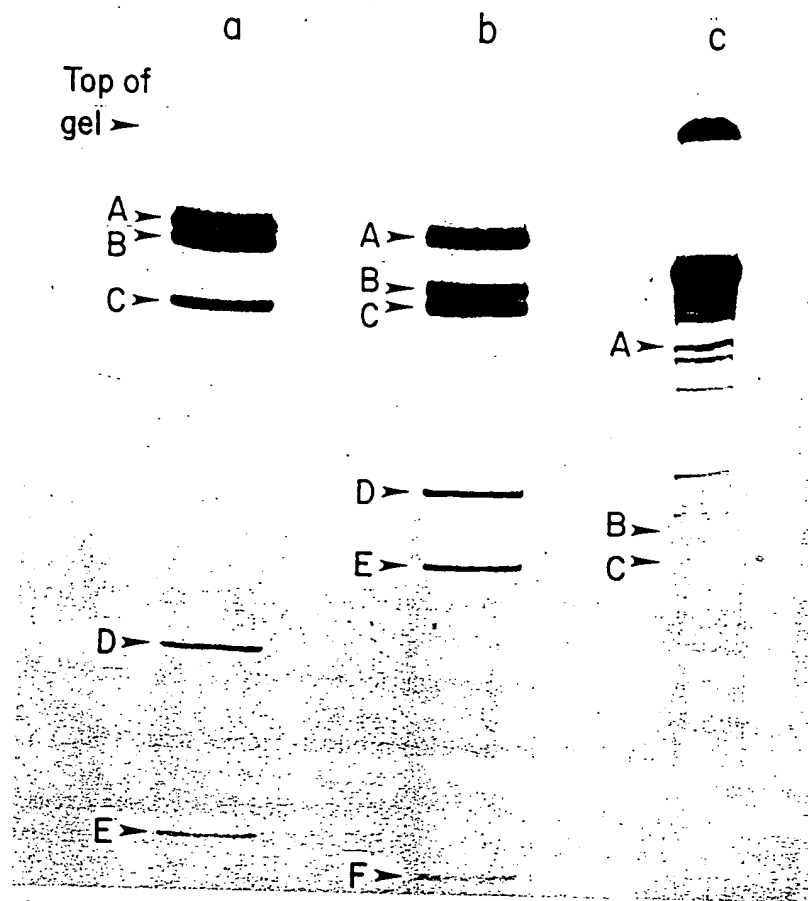


FIG. 2. Autoradiographic analysis of complete digests of SV40 DNA with *HincII* and *HindIII* and a partial digest with *HindIII*. (a) SV40 [32 P]DNA (0.1 μ g) was digested with 0.25 unit of *HincII* in 10 μ l of 10 mM Tris-HCl, pH 7.9, 7 mM $MgCl_2$, 60 mM NaCl, 7 mM 2-mercaptoethanol, 0.5 mg/ml gelatin for 1 hr at 37°. A 5- μ l aliquot was electrophoresed on a 13-cm long 4% polyacrylamide gel at 120 V for 2.5 hr and a dried-gel autoradiogram was prepared. (b) SV40 [32 P]DNA (0.1 μ g) was digested with 0.25 unit of *HindIII* in 10 μ l of 7 mM Tris-HCl, pH 7.4, 7 mM $MgCl_2$, 50 mM NaCl, 0.5 mg/ml gelatin for 1 hr at 37°. A 5- μ l aliquot was analyzed as described for sample a. (c) SV40 [32 P]DNA (4 μ g) was digested with 7.5 units of *HindIII* in a volume of 360 μ l. At 10, 20, and 35 minutes 120 μ l of the sample was removed and the reaction stopped by addition of SDS to a final concentration of 1% (w/v). A mixture of 1 μ l from each sample was electrophoresed on a 4% polyacrylamide gel at 75 V for 20 hr adjacent to a *HindIII* complete digest marker and a dried-gel autoradiogram was prepared. Fragments are labeled alphabetically in order of decreasing size.

HindIII, the positions of the final products A, B, and C indicated by arrows. In theory, an incomplete digest of circular SV40 DNA with *HindIII* might contain up to thirty partial digestion products, including the

HindIII
product

A
B
C
D
E
F

^a To account for pairs in

^b Fiers *et al.*

^c Reddy *et al.*

six unit-length
the example

Individual
purified by
appropriate
by electrophoresis
In each case
adjacent to
sured. Examination
digestion a
equimolar
autoradiogram
HindIII partial
redigested
f). One can
F in the SV40
to A, B, C
B and C.
actually a
in the preparation
comprised
tion may
digestion
conclusion
fragment

TABLE I
SIZES OF SV40 DNA FRAGMENTS PRODUCED BY CLEAVAGE
WITH *Hind*III AND WITH *Hinc*II

<i>Hind</i> III product	Nucleotide pairs ^{a-c}	<i>Hinc</i> II product	Nucleotide pairs ^{a-c}
A	1768	A	1961, ^b 1963 ^c
B	1169	B	1538
C	1099, ^b 1101 ^c	C	1067
D	526	D	369
E	447	E	240
F	215	F	29
		G	20

^a To account for the staggered breaks produced by *Hind*III, the number of nucleotide pairs in each fragment was taken to be one-half of the total nucleotides.

^b Fiers *et al.*²²

^c Reddy *et al.*²³

six unit-length linear species. Of these, seven are clearly resolved in the example, and one short fragment migrated off the gel.

Individual products of partial digestion with *Hinc*II and *Hind*III, purified by the method described in Section I,C, were redigested with the appropriate enzyme, and the products derived from each were identified by electrophoresis of the digest in parallel with a complete digest marker. In each case, the intact partial digestion product was also electrophoresed adjacent to the marker so that the distance of migration could be measured. Examples of electrophoretic analysis of partial products of *Hind*III digestion are shown in Fig. 3. When a partial product gives rise to a set of equimolar fragments, as judged from the intensities of the bands in the autoradiogram, analysis is usually straightforward. For example, the *Hind*III partial product in lane a of Fig. 3 yields fragments C and E when redigested (lane c), and the partial product in lane d yields E and F (lane f). One can conclude that C is contiguous to E and that E is contiguous to F in the SV40 genome. On the other hand, the fragment in lane g gives rise to A, B, C, and D, but clearly A and D are present in greater amount than B and C. Such a result is expected when the partial digestion product is actually a mixture of two different fragments that happened to comigrate in the preparative gel. Thus, in this example, one of the partial products is comprised of A and D, and the other of B and C. In other cases, redigestion may result in no apparent change in mobility of a putative partial digestion product because the fragment is actually a final product. This conclusion is confirmed if the putative partial product comigrates with a fragment in the complete digest marker.

with *Hinc*II and
tested with 0.25
NaCl, 7 mM
phoresed on a
adiogram was
in 10 μ l of 7
at 37°. A 5- μ l
digested with
the sample was
f 1% (w/v). A
le gel at 75 V
adiogram was

icated by
DNA with
luding the



FIG. 3. Analysis of *Hind*III partial digestion products. Lanes b, e, and h are *Hind*III complete digest markers. Lane c is the result of redigestion of the partial digestion product in lane a; lane f is the digest of the partial product in lane d; and lane i is the digest of the partial product in lane g. Each partial digestion product was digested with *Hind*III by incubating 0.01 μ g of DNA with 0.1 unit of enzyme in a volume of 30 μ l for 1 hr at 37°. Samples were electrophoresed at 120 V for 2.5 hr on a 4% polyacrylamide gel and analyzed by dried-gel autoradiography.

Qualitative results based on comigration should be verified by comparing the size of each partial digestion product with the sum of sizes of the fragments derived from it. This is particularly important for identifying instances in which two partial digestion products that comigrate are also equimolar. In contrast to the example shown in Fig. 3 (lanes d and f), the two sets of final products derived from an equimolar mixture of partial products cannot be distinguished on the basis of intensities of the bands in the autoradiogram. However, the combined sizes of all the final products derived from such a mixture will be twice the estimated length of the putatively homogeneous partial product. Although a limited amount of information can be derived from analysis of an equimolar mixture of partial products, one can usually obtain sufficient data from less ambiguous cases to construct a physical map.

The length of a partial digestion product can be estimated on the basis of electrophoretic mobility,^{1,24} using a plot of relative mobility versus log of fragment length. Figure 4 illustrates such a curve for unit-length linear SV40 DNA and the fragments in a *Hind*III digest of SV40 DNA, the

²⁴ A. J. Shatkin, J. D. Sipe, and P. Loh, *J. Virol.* 2, 989 (1968).

FIG. 4. Stannous chloride reduction of DNA with *Hinc*

fragment length genome.^{22,23} However, the relationship can be determined by summing that intensity in individual preparations; 0.2 ml of 30% scintillation fluor for aqu

Table II products of each partial the sum of length of each

²³ For example

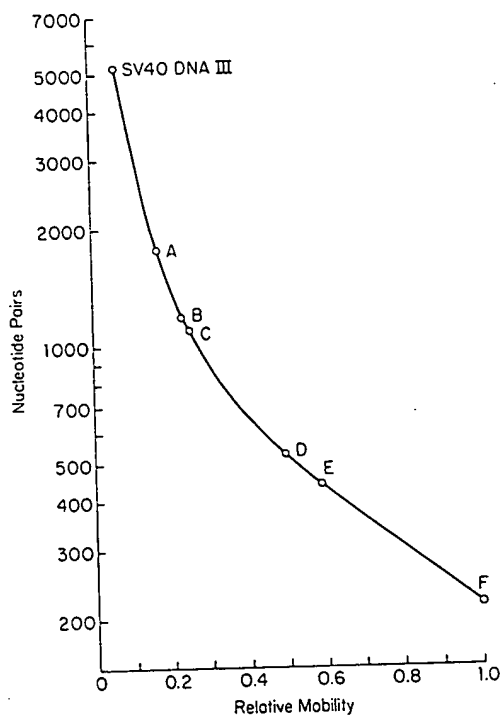


FIG. 4. Standard plot of relative mobility versus log of fragment length in a 4% polyacrylamide gel. Unit-length linear SV40 DNA and the fragments in a complete digest of SV40 DNA with *Hind*III were used as markers.

fragment lengths taken from the nucleotide sequence of the SV40 genome.^{22,23} If DNA fragments of known length are not available as markers, the relative sizes of the fragments in a complete digest of [³²P]DNA can be determined from the relative radioactivities of the fragments assuming that radioactivity is directly proportional to length. The radioactivity in individual fragments can be measured by excising the bands from a preparative gel, as described in Section I, C. Each segment is dissolved in 0.2 ml of 30% hydrogen peroxide by incubation at 65° in a tightly capped scintillation vial and counted in a liquid scintillation spectrometer using a fluor for aqueous samples.²⁵

Table II lists the results from analyses of several partial digestion products of both *Hind*III and *Hinc*II. Included are the estimated length of each partial digestion product, the final products derived from each, and the sum of lengths of the final products. For these data, the estimated length of each partial product agrees reasonably well with the sum of sizes

²⁵ For example, Aquasol, manufactured by New England Nuclear, Boston, Massachusetts.

TABLE II
ANALYSIS OF *Hind*III AND *Hinc*II PARTIAL DIGESTION PRODUCTS

<i>Hind</i> III product (relative mobility) ^a	Estimated size ^b (nucleotide pairs)	Final products	Sum of sizes ^c (nucleotide pairs)
0.39	670	E, F	662
0.18	1600	C, E	1546
0.14	2300	{ B, C A, D	2268
0.13	2500	A, D, F	2294
0.12	2900	B, C, D	2509
			2794

<i>Hinc</i> II product (relative mobility) ^a	Estimated size ^b (nucleotide pairs)	Final products	Sum of sizes ^c (nucleotide pairs)
2.2	60	F, G	49
0.19	1500	C, D	1436
0.18	1600	B, G	1558
0.15	2000	A, F	1990
0.14	2300	B, D, E	2147
0.11	3200	A, C, F	3057

^a Relative mobility was measured as distance migrated by fragment divided by distance migrated by *Hind*III F on the same gel.

^b Estimated from electrophoretic mobility, using the plot in Fig. 4.

^c The size of each final product was derived from the data of Fiers *et al.*²²

of the final products. Each set of final products listed in Table II represents a group of fragments contiguous in the original DNA molecule. For example, based on the *Hind*III partial product of relative mobility 0.39, the final product E is contiguous to F in SV40 DNA. Analysis of the partial product of relative mobility 0.18 indicates that C and E are contiguous. Because these two groups of fragments share fragment E, they can be linked in the order C-E-F. The results in Table II can be arranged such that members common to each group of contiguous fragments are placed in overlapping positions, as shown in Fig. 5, to determine the order of all the fragments. The data thus lead to the construction of the two cleavage maps in Fig. 6, one for *Hind*III and the other for *Hinc*II.

III. Ordering of Fragments through Multiple Enzyme Digestion

One application of multiple enzyme digestion for ordering fragments²⁶ parallels the use of partial digests discussed in Section II. That is, if the products of enzyme α are to be ordered, purified products of several

²⁶ R. C. Yang, A. Van de Voorde, and W. Fiers, *Eur. J. Biochem.* 61, 119 (1976).

FIG. 5. Results of contiguous fragments to determine the order

accessory enzymes. The cleavage products include three fragments. If then one can determine the cleavage site. More sufficient number of techniques are

1. Electrophoresis of restriction endonucleases



FIG. 6

[53]

[53]

PRODUCTS

Sum of sizes^c
(nucleotide pairs)

662

1546

2268

2294

2509

2794

Sum of sizes^c
(nucleotide pairs)

49

1436

1558

1990

2147

3057

divided by distance

et al.²²

Table II represents a molecule. For mobility 0.39, Analysis of the and E are contiguous fragments are determine the order of the two or *HincII*.

restriction

fragments²⁶. That is, if the products of several 119 (1976).

HindIII PRODUCTS

E C

C B

C B D

D A

D A F

F E

- E C B D A F E -

HincII PRODUCTS

C A F

A F

F G

G B

B E D

D C

- C A F G B E D C -

FIG. 5. Results of analysis of *HindIII* and *HincII* partial digestion products. Each group of contiguous fragments has been arranged so that members common to each group overlap to determine the complete order.

accessory enzymes (β , γ , δ , . . .) may be used in place of partial digestion products. For example, a fragment produced by enzyme β that includes three cleavage sites for enzyme α will yield, upon digestion with α , two α fragments and two fragments originating from the ends of the β fragment. If the end fragments can be distinguished from all α products, then one can conclude that the two observed α fragments are contiguous. Since the method requires that the β fragment include three or more α cleavage sites, accessory enzymes that produce large fragments are most useful. Moreover, several accessory enzymes are required to establish a sufficient number of sets of contiguous fragments to generate a map. The techniques required are similar to those utilized in Section II.

1. Electrophoretic profiles of fragments produced by several endonucleases (α , β , γ , δ , . . .) are determined.

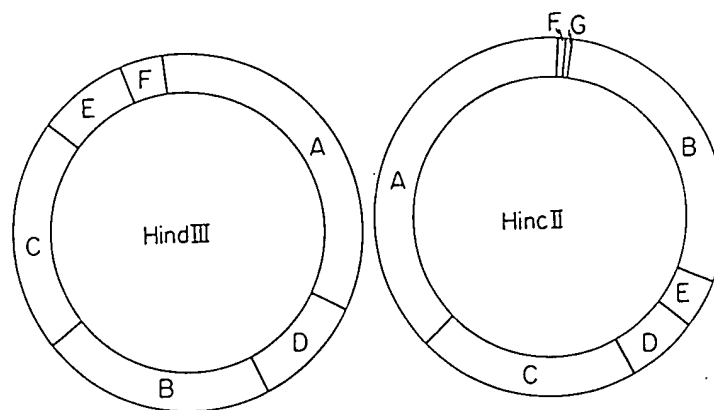


FIG. 6. Cleavage maps for the *HindIII* and *HincII* products of SV40 DNA.

2. Fragments produced by complete digestion with accessory enzymes β , γ , δ , . . . , are purified by the procedure described in Section I,C.

3. Each purified fragment is digested with enzyme α , and the limit products of α derived from each are identified by electrophoresis in parallel with a marker of α products.

4. Sets of contiguous fragments are arranged such that common members are placed in overlapping positions to construct a map.

This basic approach can also be used to correlate two existing cleavage maps. For example, the *HincII* and *HindIII* maps in Fig. 6 might be aligned by digesting purified *HincII* products with *HindIII*. The relative positions of the cleavage sites for the two enzymes can be deduced from the resulting data.

A second application of multiple enzyme digestion allows the correlation of independently constructed cleavage maps of two different enzymes. In contrast to the first approach, this method relies on electrophoretic analysis of double digests and usually requires no purification of fragments. A particularly simple analysis results if each accessory enzyme used in this method recognizes only a single cleavage site in the DNA.

The first step of the procedure involves characterizing the cleavage products of each accessory enzyme. In the model study, the endonucleases *TaqI* and *BamHI* are used to relate the *HindIII* and *HincII* cleavage maps that were established in Section II (Fig. 6). *TaqI* and *BamHI* each cleaves SV40 DNA at a single site, as shown in Fig. 7. The single bands in lanes a (*TaqI* digest) and b (*BamHI* digest) correspond to unit-length linear SV40 DNA. The distance between the cleavage sites for the two enzymes can be estimated as described in Section II by sequential digestion of ^{32}P -labeled SV40 DNA and quantitation of the radioactivity in the products, A and B (Fig. 7, lane c). Fragment A accounts for 58% of the total radioactivity and fragment B for 42%. Since SV40 DNA contains about 5200 nucleotide pairs, fragment A is approximately 3000 nucleotide pairs long and fragment B is about 2200 nucleotide pairs long.

The next step involves double digestion with each accessory enzyme and the enzymes *HincII* and *HindIII*. If optimal reaction conditions for two enzymes are similar, the enzymes can be used simultaneously. For example, for double digestion of SV40 DNA with *BamHI* and *HindIII*, 0.02 μg of SV40(^{32}P)DNA was incubated with 0.1 unit of *HindIII* and 0.1 unit of *BamHI* at 37°C for 1 hr in 20 μl of 7 mM Tris-HCl, pH 7.9, 7 mM MgCl_2 , 50 mM NaCl, 7 mM 2-mercaptoethanol, and 0.5 mg/ml gelatin. Likewise, *HincII* and *BamHI* can be used together. On the other hand, since conditions optimal for *TaqI* require incubation at 50° with no NaCl, sequential digestion is necessary.

FIG. 7. *TaqI*, μg) was digested with 0.1 unit of *TaqI* in 20 μl of 7 mM Tris-HCl, pH 7.9, 7 mM MgCl_2 , 50 mM NaCl, 7 mM 2-mercaptoethanol, and 0.5 mg/ml gelatin. Analysis was then as described for sample was inc

Top of
gel ➤

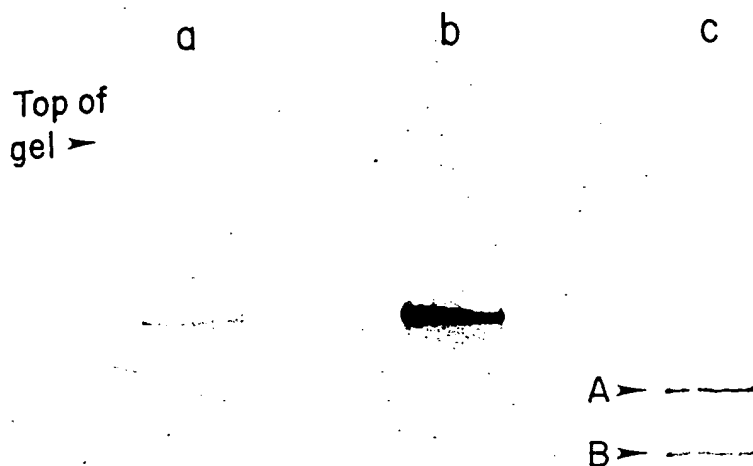


FIG. 7. *TaqI*, *BamHI*, and *TaqI/BamHI* digests of SV40 DNA. (a) SV40 [32 P]DNA (0.02 μ g) was digested with 0.1 unit of *TaqI* in 20 μ l of 10 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 0.5 mg/ml gelatin at 50° for 1 hr. The sample was electrophoresed on a 1.3% agarose gel for 3 hr at 40 V and analyzed by dried-gel autoradiography. (b) SV40 [32 P]DNA (0.02 μ g) was digested with 0.1 unit of *BamHI* in 20 μ l of 6 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 50 mM NaCl, 7 mM 2-mercaptoethanol, 0.5 mg/ml gelatin at 37° for 1 hr. Analysis was the same as for sample a. (c) SV40 [32 P]DNA (0.02 μ g) was digested with *TaqI* as described for sample a. Then 1 μ l of 1 M NaCl and 0.1 unit of *BamHI* were added and the sample was incubated at 37° for 1 hr. Analysis was the same as for sample a.

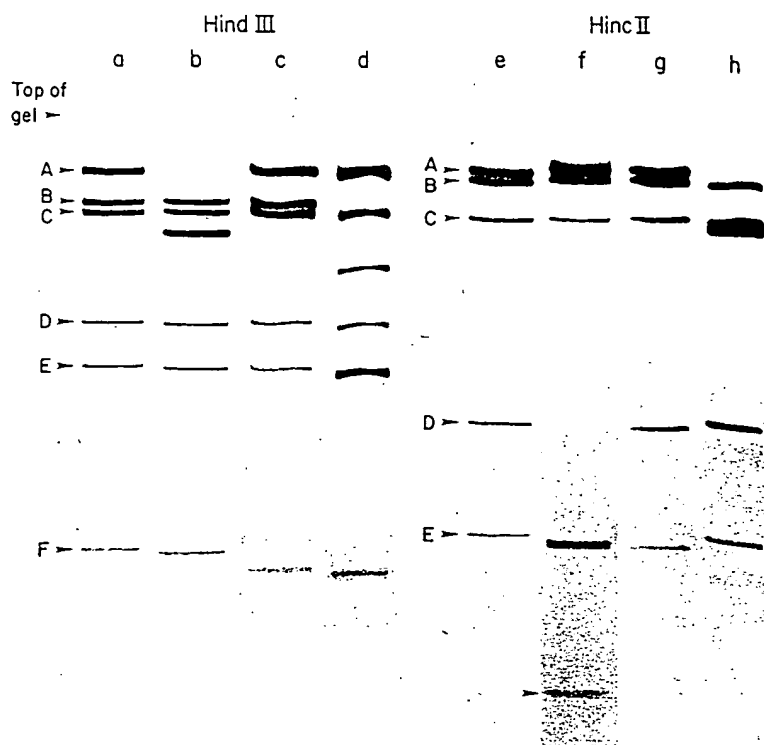


FIG. 8. Double digests of *Hind*III and *Hinc*II products with *Bam*HI and *Taq*I. Lanes a and c are complete *Hind*III digest markers; lanes e and g are complete *Hinc*II digest markers. Lane b, 0.02 μ g of SV40 [32 P]DNA was incubated with 0.1 unit of *Hind*III and 0.1 unit of *Bam*HI at 37° for 1 hr in 20 μ l of 7 mM Tris-HCl, pH 7.9, 7 mM MgCl₂, 50 mM NaCl, 7 mM 2-mercaptoethanol, 0.5 mg/ml gelatin. Lane d, 0.02 μ g of SV40 [32 P]DNA was digested with *Taq*I as for sample a in Fig. 7. One microliter of 1 M NaCl and 0.1 unit of *Hind*III were added and incubation was continued for 1 hr at 37°. Lane f, 0.02 μ g of SV40 [32 P]DNA was digested with 0.1 unit of *Bam*HI and 0.1 unit of *Hinc*II in 20 μ l of 6 mM Tris-HCl, pH 7.9, 7 mM MgCl₂, 50 mM NaCl, 7 mM 2-mercaptoethanol, 0.5 mg/ml gelatin at 37° for 1 hr. Lane h, 0.02 μ g of SV40 [32 P]DNA was digested with *Taq*I as described in the legend to Fig. 7. One microliter of 1 M NaCl and 0.1 unit of *Hinc*II were added and incubation was continued at 37° for 1 hr. All samples were electrophoresed on 4% polyacrylamide gels at 120 V for 2.5 hr and analyzed by dried-gel autoradiography.

Fragments resulting from double digestion are analyzed electrophoretically to localize the cleavage sites for *Taq*I and *Bam*HI within specific *Hind*III and *Hinc*II fragments. In Fig. 8, a comparison between a complete *Hind*III digest (lane a) and a *Bam*HI/*Hind*III double digest (lane b) indicates that in the double digest *Hind*III A is missing, and a new band below

Fragment
cleaved
by *Bam*HI

*Hind*IIIA
*Hinc*IID
*Taq*I linear DN.

Fragment
cleaved
by *Taq*I

*Hind*IIIB
*Hinc*IIA

^a Derived fr
^b Estimated

*Hind*III C i
*Hind*III B is
the cleavage
*Taq*I is withi
and h) indic
*Taq*I cleaves
the *Hinc*II I
overlap; like
results roug

In order
*Bam*HI and
mined. Each
can be estim
Fig. 4. The
model study
second poss
*Hind*III C :
pairs, about
cleotide pai
correspondi
actually a d
its center to
be reached
*Hinc*II A fi

TABLE III

Fragment cleaved by <i>Bam</i> HI	Size ^a (nucleotide pairs)	Estimated sizes ^b of products (nucleotide pairs)	
<i>Hind</i> III A	1768	900	900
<i>Hinc</i> IID	369	240	130
<i>Taq</i> I linear DNA	5224	3000	2200

Fragment cleaved by <i>Taq</i> I	Size ^a (nucleotide pairs)	Estimated sizes ^b of products (nucleotide pairs)	
<i>Hind</i> III B	1169	740	430
<i>Hinc</i> II A	1961	1000	1000

^a Derived from the data of Fiers *et al.*²²

^b Estimated from electrophoretic mobility, using the plot in Fig. 4.

*Hind*III C is present. Similarly, for the *Taq*I/*Hind*III digest (lane d), *Hind*III B is missing and a new band below *Hind*III C appears. Therefore, the cleavage site for *Bam*H1 is within the *Hind*III A fragment and that for *Taq*I is within the *Hind*III B fragment. The other two double digests (lanes f and h) indicate that *Bam*H1 cleaves within the *Hinc*II D fragment and that *Taq*I cleaves within the *Hinc*II A fragment. Since both the *Hind*III A and the *Hinc*II D fragments contain the *Bam*H1 recognition site, they must overlap; likewise, the *Hind*III B and the *Hinc*II A fragments overlap. These results roughly determine the relative orientations of the two maps.

In order to relate the two maps precisely, the exact locations of the *Bam*H1 and *Taq*I sites on the *Hind*III and *Hinc*II maps must be determined. Each double digest contains two new products, the sizes of which can be estimated on the basis of electrophoretic mobility, using the plot in Fig. 4. The simplest result of double digestion, not exemplified in this model study, is that the two new products migrate as distinct bands. A second possibility is shown in Fig. 8, lane b. The new product between the *Hind*III C and D fragments has an estimated length of 900 nucleotide pairs, about half the length of the parent fragment, *Hind*III A (1768 nucleotide pairs). The fact that the new band is more dense than the band corresponding to the longer *Hind*III C fragment indicates that the band is actually a doublet. *Bam*H1 therefore cleaves the *Hind*III A fragment near its center to produce two comigrating fragments. The same conclusion can be reached for the *Taq*I/*Hinc*II digest (lane h), in which *Taq*I cleaves the *Hinc*II A fragment (1961 nucleotide pairs) to yield two fragments, each

[53]

g h

and *TaqI*. Lanes a
lincII digest mar-
indIII and 0.1 unit
, 50 mM NaCl, 7
DNA was digested
it of *HindIII* were
40 [32P]DNA was
is-HCl, pH 7.9, 7
37° for 1 hr. Lane
end to Fig. 7. One
was continued at
at 120 V for 2.5 hr

electrophoret-
within specific
en a complete
(lane b) indi-
w band below

TABLE IV
CLEAVAGE OF FRAGMENTS A AND B FROM A *TaqI/BamHI* DIGEST
WITH *HincII* AND *HindIII*

<i>TaqI/BamHI</i> fragment	Products of digestion with <i>HincII</i>		Products of digestion with <i>HindIII</i>	
	Identifiable <i>HincII</i> products	Estimated sizes ^a of additional products	Identifiable <i>HindIII</i> products	Estimated sizes ^a of additional products
A	B, E, F, G	240 1000	C, E, F	430 900
B	C	130 1000	D	740 900

^a Estimated from electrophoretic mobility, using the plot in Fig. 4.

about 1000 nucleotide pairs in length. In contrast to these cases, the *TaqI/HindIII* and *BamHI/HincII* digests exemplify another possible result, namely, that only one new band appears but, adjudged from the intensity of the band in the autoradiogram, it cannot be a doublet. Shown in lane d of Fig. 8, the *HindIII* B fragment (1169 nucleotide pairs) is cleaved by *TaqI* to produce a new fragment about 740 nucleotide pairs long that migrates between C and D. Since the predicted length of the companion new fragment is about 430 nucleotide pairs, it should migrate near *HindIII* E (450 nucleotide pairs long). Consistent with this prediction is the observation that the *HindIII* E band is broader and denser than the D fragment in the double digest, indicating that it is indeed a doublet. Likewise, *BamHI* cleaves the *HincII* D fragment (lane f) to yield the new fast-migrating fragment (about 140 nucleotide pairs), indicated by an arrow and a second fragment that comigrates with the *HincII* E fragment (about 240 nucleotide pairs). These results are summarized in Table III.

The data are used to construct a cleavage map by comparing the known distance between the *TaqI* and *BamHI* cleavage sites with the possible distances calculated from the lengths of the double digestion products. For example, since *TaqI* produces two fragments from *HindIII* B, 740 and 430 nucleotide pairs in length, the cleavage site might be nearer the B-D junction or nearer the B-C junction (see Fig. 6). As summarized in Table III, *BamHI* cleaves the *HindIII* A fragment near its center. The shorter distance between the *BamHI* and *TaqI* cleavage sites, about 2200 nucleotide pairs, should equal the sum of half of *HindIII* A (900 nucleotide pairs), *HindIII* D (526 nucleotide pairs) and either the 740- or 430-nucleotide pair fragment derived from *HindIII* B. The former possibility yields a total of 2166 nucleotide pairs whereas the latter yields only 1856. The *TaqI* site is, therefore, near the B-C junction. These arguments locate

FIG. 9. Com
BamHI, *HindIII*

the *BamHI* a
the *HincII* m
fragments 13
ment near its
length. The
sites in the
near the D-I
so that *Bam*
results can
BamHI dou
shown in Ta
C, a new p
nucleotide p
a new prod
pairs long.
are sound.

The dou
SV40 geno
located wit
tationship be

*Bam*H1 DIGESTof digestion with *Hind*IIIEstimated sizes* of
additional products

430	900
740	900

g. 4.

o these cases, the
ther possible result,
d from the intensity
et. Shown in lane d
) is cleaved by *Taq*I
s long that migrates
ompanion new frag-
near *Hind*III E (450
is the observation
: D fragment in the
Likewise, *Bam*H1
new fast-migrating
arrow and a second
nt (about 240 nu-
III.

by comparing the
sites with the
ble digestion
om *Hind*III
it be nearer
mmarized
nter. The
out 2200
leotide
r 430-
sibility
1856.
cate

[53]

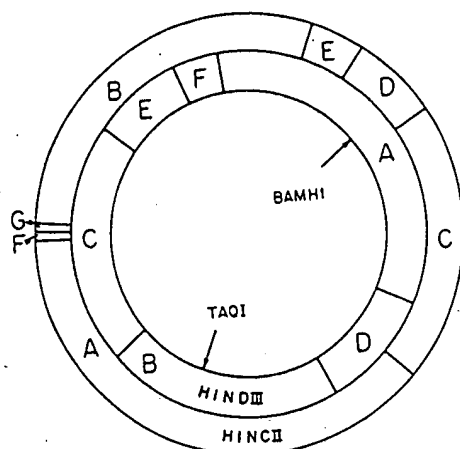


FIG. 9. Composite physical map of SV40 DNA, including the cleavage sites for *Taq*I, *Bam*H1, *Hind*III, and *Hinc*II.

the *Bam*H1 and *Taq*I cleavage sites on the *Hind*III map. With regard to the *Hinc*II map, *Bam*H1 cleaves the D fragment asymmetrically to yield fragments 130 and 240 nucleotide pairs long and *Taq*I cleaves the A fragment near its center to yield two fragments about 1000 nucleotide pairs in length. The two possible arrangements of the *Taq*I and *Bam*H1 cleavage sites in the *Hinc*II map yield a distance of either 2307 (if *Bam*H1 cleaves near the D-E junction) or 2197 (if *Bam*H1 cleaves near the D-C junction), so that *Bam*H1 probably cleaves *Hinc*II D near the D-C junction. These results can be verified by purifying fragments A and B from a *Taq*I/*Bam*H1 double digest and cleaving each with *Hinc*II and with *Hind*III. As shown in Table IV, the digestion of fragment B with *Hinc*II yields *Hinc*II C, a new product of 1000 nucleotide pairs, and a new product of 130 nucleotide pairs. Digestion of fragment B with *Hind*III yields *Hind*III D, a new product of 900 nucleotide pairs, and a third product 740 nucleotide pairs long. Thus, the conclusions drawn from the original double digests are sound.

The double digest data generate the composite physical map of the SV40 genome shown in Fig. 9. Not only are the *Taq*I and *Bam*H1 sites located within specific *Hind*III and *Hinc*II fragments, but also the relationship between the *Hind*III and *Hinc*II cleavage sites is established.